Amentoflavone prevents sepsis-associated acute lung injury through Nrf2-GCLc-mediated upregulation of glutathione

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Sepsis is a serious medical problem and is one of the main causes of high mortality in intensive care units. Fifty percent of patients with severe sepsis will develop acute lung injury (ALI). Amentoflavone (AMF) is a polyphenolic compound possessing potent anti-inflammatory activities. The study aimed to explore the protective effects of AMF against ALI in cecal ligation and puncture (CLP)-induced septic rats. The results showed that AMF administration protected against septic ALI, as reflected by marked amelioration of histological injury of lung tissues and decrease of pulmonary edema in CLP-treated rats. AMF ameliorated CLP-induced increase of systemic and lung TNFα and IL-1β and binding activity of p65 NF-κB, indicating the inhibition of inflammation. Moreover, AMF prevented CLP-induced oxidative stress, as evidenced by increase of oxygen consumption rate, decrease of TBARS content, increase of SOD activity and GSH level in lung tissue of CLP-treated rats. CLP resulted in significant decrease of mRNA expression of Nrf2 and GCLc, which was inhibited by AMF. AMF-induced protective effects on ALI, inflammation, and oxidative stress were inhibited by lentivirus shRNA-mediated silence of Nrf2 and buthionine sulphoximine (BSO), an inhibitor of GSH synthesis. AMF increased Nrf2-binding activity in GCLc promoters in lung tissue of CLP-treated rats. The results suggested that AMF protected against ALI in septic rats through upregulation of Nrf2-GCLc signaling, enhancement of GSH antioxidant defense, reduction of oxidative stress and final amelioration of inflammation and histological injury of lung. The data provide new therapeutic options for the treatment of sepsis-associated ALI.

INTRODUCTION

Sepsis is a serious systemic inflammatory syndrome that is caused by infection, blood loss, trauma, and neoplasm (Liu *et al.*, 2016). Sepsis affects millions of individuals worldwide and is one of the main causes of high mortality in intensive care units (ICU). To date, most of sepsis complications remain refractory, and development of novel effective therapeutic approaches is urgently needed. Although the mechanism of sepsis injury is not completely clear, oxidative stress has been believed to play a pivotal role (Bajcetic *et al.*, 2014; Duran-Bedolla *et al.*, 2014).

Sepsis could severely result in multiple organ dysfunction, including cardiovascular system, liver, kidney and lung. It is generally considered that damage of the lung is one of the most severe outcomes of sepsis (Andrews *et al.*, 2005). It is estimated that about fifty percent of patients with severe sepsis will develop acute lung injury (ALI) and even acute respiratory distress syndrome (ARDS), a more severe consequence of ALI (Rubenfeld *et al.*, 2005; Maybauer *et al.*, 2006). During the development of ALI/ARDS, oxidative stress and inflammatory responses were enhanced, leading to the failure of multiple organs (Dolinay *et al.*, 2012).

As a polyphenolic compound, amentoflavone (AMF) is derived from extracts of *Selaginella tamariscina* and has been found to possess potent anti-inflammatory activities (Huang *et al.*, 2012; Tsai *et al.*, 2012; Woo *et al.*, 2005; Banerjee & Valacchi *et al.*, 2002). It is also found that AMF could inhibit the expression of cytokines in lung cell lines (Banerjee & Van der Vliet *et al.*, 2002). Banerjee showed that AMF inhibited TNFα-induced cyclooxygenase-2 expression through suppression of NF-κB activation in A549 cells (Banerjee & Van der Vliet *et al.*, 2002). However, whether AMF exhibits beneficial effect on sepsis-induced ALI is not known.

Cecal ligation and puncture (CLP)-induced sepsis in animals is most frequently used as animal model of sepsis as it represents the characteristics of pathophysiology of human sepsis (Dejager *et al.*, 2011; Scarlett *et al.*, 2004). In the current study, using CLP-induced animal model of sepsis, we aimed to investigate the effect of AMF on sepsis-induced ALI, to focus on AMF-exhibited effect on inflammation and oxidative stress and to elucidate the possible mechanisms. The results showed that AMF prevented CLP-induced ALI through suppression of oxidative stress and inflammation via nuclear factor (erythroid-derived 2)-like 2 (Nrf2)/catalytic subunit of glutamate-cysteine ligase (GCLc)/gluthione (GSH) signaling.

MATERIALS AND METHODS

Chemicals and reagents. AMF and BSO were obtained from Sigma-Aldrich. TNFα and IL-1β ELISA kits were purchased from R&D Systems. TBARS and SOD assay kits were obtained from Nanjing Jiancheng Company. GSH assay kit was obtained from Biovision. ELISA-based NF-κB p65 transcription factor assay kit was purchased from Active Motif North America. CHIP as-

say kit was obtained from Abcam. RNA isolation and Real-time PCR reagents were purchased from TaKaRa. All other chemicals used were of analytical grade.

Animals. Animal experiments were conducted under the guidelines for the care and use of laboratory animals published by the National Institute of Health. Experimental protocols were approved by the Ethical Committee of Shaanxi Provincial People's Hospital on Animal Care. Male Sprague-Dawley (SD) rats (220–260 g) were obtained from the Animal Center of Xi'an Jiaotong University. Rats were kept in a temperature-controlled room with a 12 h light/12 h dark cycle for 1 week before the experiment, with free access to food and water.

Animal model of sepsis. Fifty animals were randomly divided into five groups (10 rats in each group): Sham group, CLP group, AMF group (CLP + 50 mg/kg AMF), AMF + LV-shNrf2 (CLP + 50 mg/kg AMF + LV-shRNA Nrf2), AMF + BSO (CLP + 50 mg/kg AMF + 10 mg/kg buthionine sulfoximine). CLP-induced sepsis model was built in CLP, AMF, AMF + LV-shNrf2, and AMF + BSO groups as previously described (Liu *et al.*, 2016). In brief, after fasting overnight, rats were anesthetized using 10% chloral hydrate (3 ml/kg, intraperitoneal). After sterilization, a 1.5-cm incision was cut in ventral midline of abdomen and then the cecum was gently exposed and ligated with a 3–0 silk and punctured with an 18-gauge needle at three locations. After that, the cecum was repositioned and the abdomen was closed. The same sham surgery and cecum manipulation was conducted in Sham rats without ligation and puncture. Rats in AMF + LV-shNrf2 group were treated intranasally with either 4×10^7 PFUs (in 50 µl) letivirus shRNA targeting Nrf2 (Shanghai GenePharma Co., Ltd, China) 24 hours before the surgery and rats in the other groups were treated with scramble shRNAs. Rats in AMF + BSO group were intraperitoneally injected with 10 mg/kg BSO 2 hours before the surgery and rats in the other groups were injected with vehicle. Rats in AMF, AMF + LV-shNrf2, and AMF + BSO groups were intraperitoneally administrated with 50 mg/kg AMF 6 hours before the surgery and rats in the other groups were given with vehicle (0.1% Tween 80). Saline (2 ml/100 g body weight) was given subcutaneously to the rats immediately after the operation for resuscitation. No antibiotics were administered. AMF was administrated daily for 5 consecutive days. 6 days after the surgery, rats were euthanized. Blood samples were collected. Left lung was excised for wet/dry ratio analysis. Part of right lung was excised for histological examination. Part of right lung was homogenized in a phosphate buffer solution for further biochemical analysis. Part of right lung was stored at –70°C for real-time PCR detection.

Histological examination. Lung tissues were fixed in 4% paraformaldehyde overnight, dehydrated, embedded, and sliced into 5-µm sections. After that, sections were stained with hematoxylin and eosin, and were observed under a light microscopy (Olympus, Tokyo, Japan).

Lung wet/dry weight ratio. Wet weight of left lung was recorded immediately after the excision. Then the lung tissues were placed in an incubator at 70°C for 48 h and the dry weight was recorded. The wet to dry weight ratio was calculated as follows: wet weight–dry weight/dry weight.

Determination of inflammation. Levels of tumor necrosis factor-alpha (TNFα) and interleukin-1β (IL-1β) in serum and lung homogenates were determined by ELISA kits according to the manufacturer's instructions. Nuclear protein was extracted and DNA-binding activity of p65 NF-κB was determined using an ELISA-based

NF-κB p65 transcription factor assay kit according to the manufacturer's protocols. In each assay, a standard curve and positive/negative controls were included. The absorbance at 450 nm was measured using a microtiter ELISA reader (BioRad, USA). The assay sensitivity for IL-1β was <2 pg/mL and for TNF-α was 0.5–5.5 pg/mL.

Oxygen consumption rate. Mitochondrial function in lung tissue was assessed by the rate of oxygen consumption. In brief, mitochondria were isolated from lung tissues according to the manufacture's protocols (Thermo scientific, USA). Mitochondria were isolated using differential centrifugation procedures. The resulting pellets were suspended in Tris buffer and mitochondrial marker cytochrome c was detected to evaluate the purity of mitochondria. A Clark-type Oxygen Electrode was used to measure oxygen consumption rate. Respiration was initiated by addition of 2 mM pyruvate + 5 mM malate or 5 mM succinate. After 2 min, state 3 respiration was induced by adding 1 mM ADP. The results were expressed as percentage of oxygen consumption.

Determination of oxidative stress. TBARS levels and SOD activities in lung homogenates were detected by commercial kits (Nanjing Jiancheng Company, China). TBARS content was determined by the TBA reaction. The reaction mixture was heated at constant temperature at 95°C for 40 min, cooled by running water and then centrifuged at 3500 r/min for 10 min. The absorbance at 532 nm was measured and the results were shown as µmol/mg protein. SOD activity was measured through detection of nitroblue tetrazolium reduction at 450 nm. One unit of SOD activity was defined as the amount of enzyme required to inhibit the oxidation reaction by 50 % and was expressed as U/mg protein. GSH content in lung homogenates was determined by a commercial assay kit (Biovision, USA). GSH was detected by the specific reaction of o-phthalaldehyde with GSH (not GSSG). The fluorescence of the product was measured at Ex/Em 340/420 nm. The results were shown as nmol/mg protein.

Real-time PCR. Total RNA was isolated from lung tissues using the TRIzol reagent. 500 ng total RNA was transcribed into cDNA using a commercial kit (TaKaRa, Japan) according to the manufacturer's instructions. Real-time PCR was performed in a 20 µl reaction mixture containing 10 µl $2 \times$ SYBR green master mix, 7 µl H_2O, 2 µl primers (10 µM), and 1 µl template DNA. Cycling was conducted using a quantitative PCR instrument (BIORAD, USA) and a SYBR® Premix Ex Taq™ kit (TaKaRa, Japan) were used to detect target gene expressions, and β-actin was used as an internal reference. The $2^{-\Delta\Delta CT}$ method was employed to determine the relative expression of target genes normalized to β-actin, and experiments were repeated in triplicate.

Chromatin immunoprecipitations. Chromatin immunoprecipitations (CHIP) was conducted to evaluate the transcriptional activity of Nrf2 in the regulation of GCLc. Chromatin of tissues prepared according to instructions of a kit for chromatin preparation from tissues for ChIP (Abcam, USA). Immunoprecipitations were carried out using anti-Nrf2 antibody (Cell Signaling Technology, USA) or normal mouse IgG (Abcam). Real-time PCR was performed and the results were normalized to the respective input signals and expressed as folds of sham.

Statistical analysis. Results were expressed as means ± S.E.M. Graph-Pad Prism software was used to perform the statistical analysis using one-way ANOVA followed by Newmane Keuls multiple-comparison post hoc
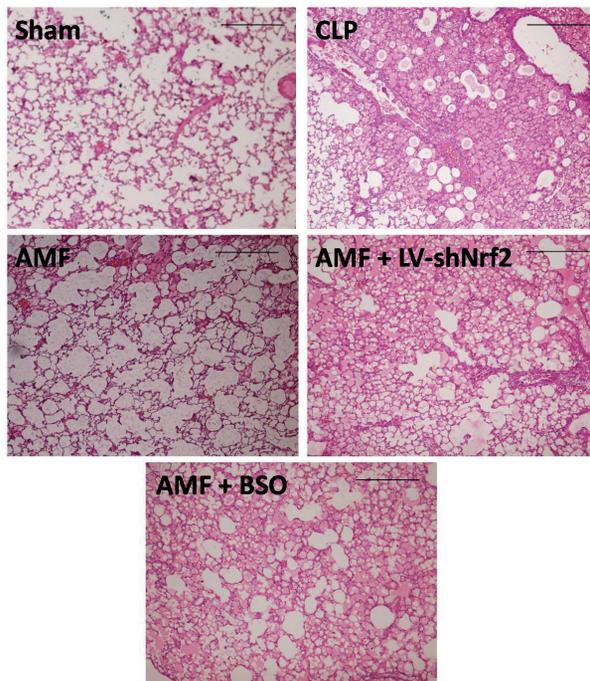
Figure 1. Effect of AMF on histological injury of lung tissue in CLP-treated rats.
CLP-induced sepsis model was built in rats and some rats were pre-administrated with AMF or LV-shNrf2 or BSO before the surgery. After the treatment, histological injury of lung tissues was measured by HE staining.

test. P value < 0.05 was considered to be statistically significant.

RESULTS

AMF ameliorated CLP-induced histological injury in lung tissues

To evaluate the effect of AMF on CLP-induced injury in lung tissues in rats, histological examination was performed. As shown in Fig. 1, we showed that there was severe hemorrhage, alveolar congestion, thickening of alveolar wall, and infiltration and aggregation of neutrophils in alveoli and vessel walls in the lung of CLP-treated mice, indicating that CLP resulted in significant inflammatory response. The administration of AMF notably inhibited these inflammatory changes in rats. However, the treatment of letivirus of shNrf2 or BSO, a glutathione (GSH) synthesis inhibitor, markedly inhibited AMF-induced improvement of inflammatory response in lung tissues.

AMF ameliorated CLP-induced pulmonary edema

Wet/dry weights ratio of lung tissue was detected. In Fig. 2, we show that CLP induced a significant increase of wet/dry weights ratio of lung tissue, indicating that CLP resulted in a marked pulmonary edema. AMF administration markedly inhibited the increase of wet/dry weights ratio of lung tissue in CLP-treated rats. In contrast, the treatment of shNrf2 letivirus or BSO markedly suppressed AMF-induced amelioration of pulmonary edema in CLP-treated rats.
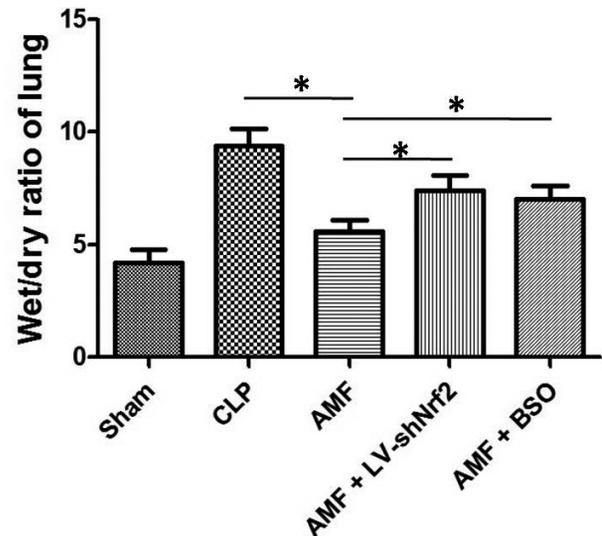

Figure 2. Effect of AMF on wet/dry weights of lung tissue in CLP-treated rats.
After the treatment, wet and dry weights of lung tissues were weighed and wet/dry raio of lung tissue was calculated (p=0.0002). *p<0.05, statistical difference between the two groups.

AMF ameliorated CLP-induced inflammation

To evaluate the effect of AMF on inflammation in CLP-treated rats, levels of cytokines and activity of p65 NF-$\varkappa$B were measured. Systemic and lung levels of TNFα and IL-1β were determined by ELISA kits and the results showed that serum and lung TNFα and IL-1βlevels were significantly increased by CLP (Fig. 3A_C and D). Moreover, p65 NF-$\varkappa$B binding activity was markedly increased by CLP (Fig. 3E). AMF administration markedly inhibited CLP-induced increase of serum and lung TNFα and IL-1β levels and p65 NF-$\varkappa$B binding activity (Fig. 3). shNrf2 letivirus or BSO treatment could suppress AMF-induced inhibition of serum and lung TNFα and IL-1β levels and p65 NF-$\varkappa$B binding activity in CLP-treated rats (Fig. 3). The results indicated that AMF administration prevented CLP-induced inflammation in lung tissue which was inhibited by shNrf2 or BSO.

AMF ameliorated CLP-induced oxidative stress

To evaluate the effect of AMF on oxidative stress in rats treated by CLP, mitochondrial function and several oxidation-sensitive markers were determined. Mitochondria are main targets of oxidative injury and mitochondrial function is a sensitive indicator of oxidative stress status. In Fig. 4A, we showed that oxygen consumption rate in lung tissues treated by CLP was significantly decreased. AMF markedly increased oxygen consumption rate in lung tissues of CLP-treated rats (Fig. 4A). AMF-induced effect on mitochondrial function was inhibited by knockdown of Nrf2 and the treatment of BSO (Fig. 4A). As shown in Fig. 4B, C and D, CLP resulted in a significant increase of thiobarbituric acid reactive substances (TBARS) content, a marked decrease of superoxide dismutase (SOD) activities and GSH levels in lung tissues. AMF markedly prevented CLP-induced changes of oxidative stress markers in lung tissues which effect was inhibited by shRNA of Nrf2 and BSO (Fig. 4B, C and D). The results demonstrated that AMF administration prevented CLP-induced oxidative injury in
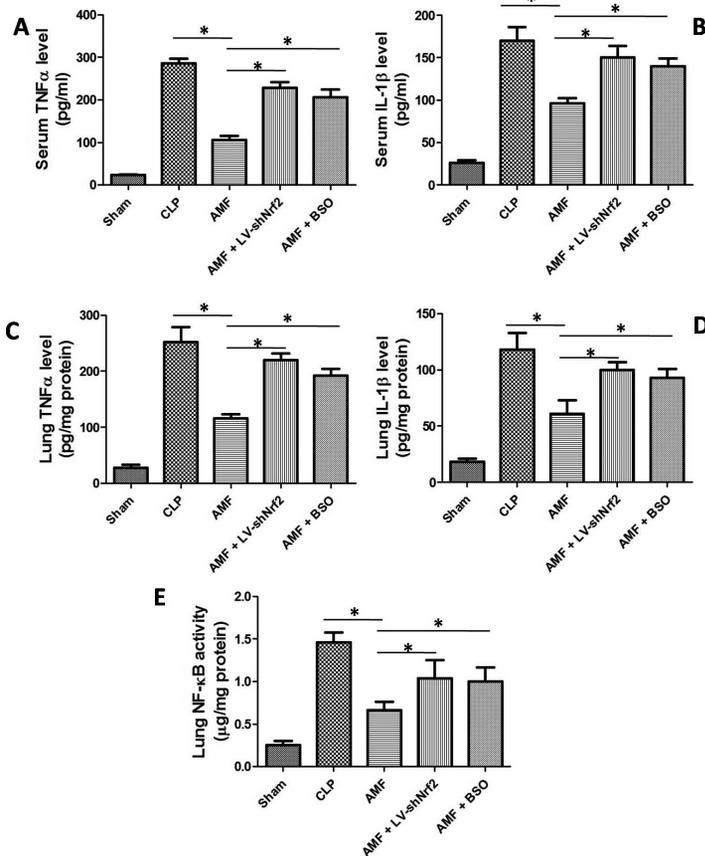
Figure 3. Effect of AMF on inflammation in lung tissue in CLP-treated rats.
Serum (A and B) and lung (C and D) TNFα (A and C) and IL-1β (B and D) levels were detected using commercial ELISA kits. Results were expressed as pg/ml or pg/mg protein. *P*<0.001 in these experiments. (E) p65 NF-κB binding activity was determined by an ELISA kit. Results were expressed as µg/mg protein (*p*<0.001). *p*<0.05, statistical difference between the two groups.
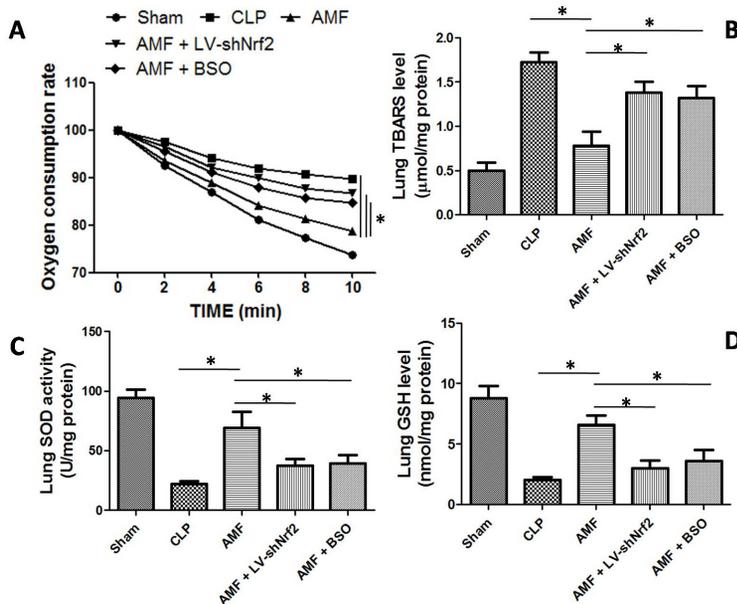

Figure 4. Effect of AMF on oxidative stress in lung tissue in CLP-treated rats.
After the treatment, (A) mitochondria in lung tissue were isolated and oxygen consumption rate was measured. Results were shown as percentage of oxygen consumption rate (*p*=0.0003). TBARS content (B), SOD activity (C) and GSH level (D) in lung tissue homogenates were determined by commercial assay kits. Results were expressed as µmol/mg protein (*p*<0.001), U/mg protein (*p*<0.001) and nmol/mg protein (*p*<0.001), respectively. *p*<0.05, statistical difference between the two groups.

lung tissue and shNrf2 and BSO could inhibit the antioxidative effect of AMF.

AMF prevented CLP-induced decrease of Nrf2-GCLc signaling

Considering the pivotal role of GSH in the antioxidant defense system, we further explored the mechanism of AMF-induced regulation of GSH. Expression of GCLc, the rate-limiting enzyme for GSH synthesis, was determined. As shown in Fig. 5A, mRNA expression of GCLc was significantly decreased by CLP. AMF markedly inhibited CLP-induced decrease of GCLc mRNA expression (Fig. 5A). shNrf2, but not BSO, resulted in a significant inhibition of AMF-exhibited suppression of CLP-induced decrease of GCLc mRNA expression (Fig. 5A). To further explore the possible mechanism underlying AMF-induced regulation of GCLc expression, expression and activity of Nrf2, a central transcription factor controlling redox balance, were measured. In Fig. 5B, we showed that AMF induced a significant inhibition of CLP-resulted decrease of Nrf2 mRNA expression. The effect of AMF on Nrf2 mRNA expression in CLP-treated rats was suppressed by shRNA of Nrf2, but not BSO (Fig. 5B). Moreover, CLP resulted in a notable decrease of Nrf2-binding in GCLc promoters which effect was prevented by AMF administration (Fig. 5C). shRNA of Nrf2, but not BSO, suppressed AMF-induced increase in Nrf2-binding activity in GCLc promoters (Fig. 5C). The results demonstrated that regulation of GCLc by Nrf2 may be involved in AMF-induced protective effects against CLP-resulted GSH depletion and lung injury.

DISCUSSION

CLP-induced sepsis model is a widely accepted animal model for the research of sepsis-associated mechanism and the investigation of potential therapeutic options for the treatment of sepsis. In the current study, using CLP-induced septic rats, we investigated the protective effects of AMF against sepsis-associated ALI. We found that AMF administration protected against septic ALI, as reflected by marked amelioration of histological injury of lung tissues and decrease of pulmonary edema in CLP-treated rats.

It is generally believed that inflammation is the fundamental pathological process in the pathogenesis of organ injury in sepsis (Dounousi *et al.*, 2016; Hahn *et al.*, 2016). Especially for ALI, inhibition of inflammation is considered to be a potent therapeutic strategy (Xie *et al.*, 2012; Lin *et al.*, 2015). It has been found
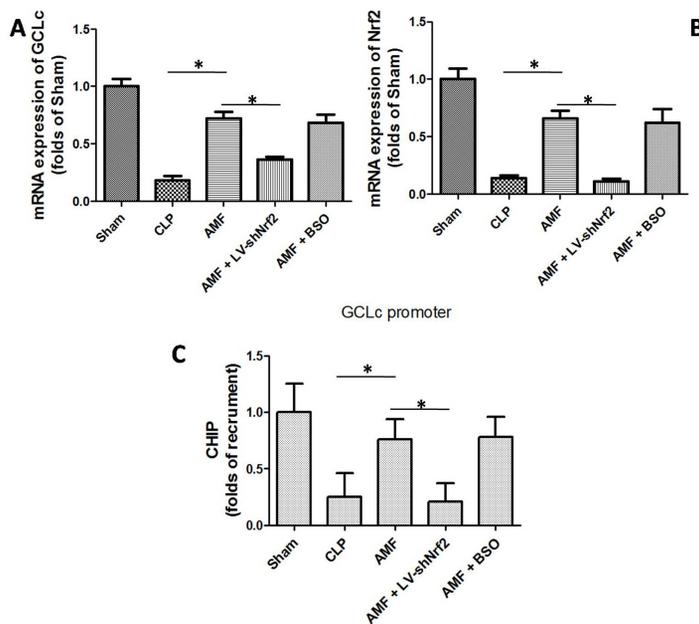
Figure 5. Effect of AMF on the regulation of GCLc by Nrf2 in lung tissue in CLP-treated rats.
mRNA expression of GCLc (**A**) and Nrf2 (**B**) in lung tissues was determined by real-time PCR. Results were shown as folds of sham group. (**A**) p=0.003; (**B**) p=0.002. The binding activity of Nrf2 to GCLc promoters was determined by CHIP assay (**C**) and results were shown as folds of sham (p=0.007). *p<0.05, statistical difference between the two groups.

that proinflammatory cytokines, such as TNFα and IL-1β, are increased in the serum of septic patients (Kang *et al.*, 2001a). NF-κB is a central transcription factor in the regulation of inflammation through controlling various proinflammatory gene expression, including TNFα, IL-1β, IL-6, and IL-8 (Cortez *et al.*, 2013). NF-κB-mediated inflammation plays an important role in the pathogenesis of ALI and sepsis (Kang *et al.*, 2001b). p65 is a major sub-member of NF-κB that mediates its proinflammatory effect. Consistent with previous literature, we found that CLP resulted in severe inflammation, as illustrated by increase of systemic and lung TNFα and IL-1β levels and the binding activity of NF-κB. The administration of AMF significantly inhibited these changes induced by CLP, indicating the amelioration of inflammation.

To explore the mechanism of the protective effect of AMF against CLP-induced inflammation and ALI, redox status was measured in the study. We found that AMF administration notably inhibited CLP-induced decrease of oxygen consumption, increase of TBARS content, decrease of SOD activity and GSH level, indicating that AMF inhibited CLP-induced oxidative stress. Oxidative stress is defined as imbalance between reactive oxygen species (ROS) generation and antioxidant defense (Avogaro *et al.*, 2008). Damage of antioxidant system could result in excessive accumulation of ROS, leading to oxidative injury of various macromolecules and important organelles (Avogaro *et al.*, 2008). Oxidative stress is closely related with inflammation and contributes to sepsis-associated injury (Zolali *et al.*, 2015; Gerin *et al.*, 2016). Mitochondrial function is a sensitive indicator of intracellular redox state. TBARS indicates the content of malondialdehyde (MDA), an end product of lipid peroxidation (Napierala *et al.*, 2016). SOD is an important antioxidant enzyme catalyzing the dismutase of superoxide into hydrogen peroxide (Shi *et al.*, 2016). GSH is one of the most important antioxidant proteins, functioning to eliminate ROS directly or indirectly under the catalysis of

antioxidant enzymes (Couto *et al.*, 2016). We should point out that the markers of oxidative stress determined in the study, including TBARS, SOD, and GSH, are indirect markers. Only the antioxidant enzyme SOD was determined to evaluate the antioxidant defense. In future study, more direct markers, such as ROS concentration, and antioxidant enzymes are needed to accurately evaluate the redox status. Even so, based on previous literature and our results, we demonstrated that the antioxidative activity of AMF was involved in the protective effects against sepsis-associated ALI.

To further explore the molecular mechanism of AMF-exhibited antioxidative activity, regulation of GSH synthesis by AMF was evaluated. GCLc is reported to be the rate-limiting enzyme for the synthesis of GSH under the transcriptional regulation of Nrf2 (Jiang *et al.*, 2016; Takahashi *et al.*, 2016; Kuwano *et al.*, 2015). Activation of Nrf2 and its target gene GCLc was show to protect against LPS-induced inflammatory response (Thimmulappa *et al.*, 2007; Thimmulappa *et al.*, 2006). In the present study, we showed that knockdown of Nrf2 by lentivirus and inhibition of GSH synthesis by BSO significantly suppressed AMF-induced inhibitory effect on histological injury, pulmonary edema, inflammatory response and oxidative stress in the development of ALI in septic rats. We also showed that AMF increased the binding activity of Nrf2 in the promoters of GCLc. The results demonstrated that enhancement of GSH through Nrf2-regulated GCLc was involved in the protective effects of AMF. Further studies are needed to explore whether Nrf2 is a direct target of AMF and to examine the structure-activity relationship. Since the mRNA expression of Nrf2 was altered, it was indicated that AMF could activate the upstream transcriptional regulator of Nrf2 which needed further efforts to elucidate. Moreover, the results also showed that knockdown of Nrf2 and inhibition of GSH synthesis did not completely reversed the beneficial effect of AMF, indicating that additional mechanism may be involved in the protective effect of AMF against sepsis. Furthermore, we should notice that AMF could not completely prevent sepsis-associated injury. Therefore, AMF can be used as a promising therapeutic option in combination with additional agents in the prevention and treatment of sepsis and its complications.

In conclusion, in the current study, we found that AMF protected against ALI in septic rats through upregulation of Nrf2-GCLc signaling, enhancement of GSH antioxidant defense, reduction of oxidative stress and final amelioration of inflammation and histological injury of lung. The data provide new therapeutic options for the treatment of sepsis-associated ALI.

Conflict of interest

The authors declare none.

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